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Short communication

Troubleshooting carry-over of LC–MS/MS method for rifampicin, clarithromycin and metabolites in human plasma

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ABSTRACT

Clarithromycin and rifampicin are used for the treatment of Mycobacteria. Pharmacokinetic drug interaction is possibly due to the influence of the two drugs on the liver enzymes. Using a Hypurity Aquastar C18 column (50 mm × 2.1 mm × 5 μm) for liquid chromatography including a polar end-capped phase for the determination of clarithromycin, rifampicin and their metabolites together in plasma using LC–MS/MS resulted in a substantial carry-over. As a consequence, the throughput of the method is not assured. Using a step-by-step troubleshooting procedure, such carry-over was found originating from column memory effect. With the use of another type of C18 column, the carry-over is eliminated. Due to the absence of carry-over, the analytical concentration ranges are extended and are therefore more appropriate for the analysis of patient samples. The method was re-validated for linearity, reproducibility and dilution integrity.

1. Introduction

The combination of rifampicin (RIF) and clarithromycin (CLR) can be used to improve the treatment outcome and prevent the resistance of *Mycobacteria*. It is well reported that RIF is a strong liver enzymes inductor while CLR is an inhibitor. Several small studies suggested that RIF may reduce the CLR plasma concentration while CLR, on the other hand, elevates the RIF plasma level [1,2]. For that reason, therapeutic drug monitoring of these substances may help to assure adequate drug exposure. Furthermore, the metabolism of RIF and CLR by cytochrome P450 results in active metabolites including 25-desacetylrifampicin (Dac-RIF) and 14-hydroxyclarithromycin (14OH-CLR), respectively [3,4]. The analysis of these substances is therefore recommended, but is seldom implemented in analytical methods [5,6]. Oswald et al. developed a method for simultaneous determination of CLR and RIF and their metabolites but not in human plasma [6]. The LC–MS/MS method published by van de Velde et al. could simultaneously determine CLR, RIF and their metabolites in human plasma [5]. In this method, the authors reported a persistent carry-over in the analysis of RIF and Dac-RIF which required five blank injections to eliminate if high standard or quality control sample was eluted. Because of the carry-over, the LLOQ for RIF and Dac-RIF was 0.2 mg/L which is relatively high in comparison with low plasma trough level of these substances [1,7,8]. In addition, if the concentration of RIF or Dac-RIF is higher than the upper limit of quantification (ULOQ: 5 mg/L), the sample needs to be diluted, and re-analyzed. It is noticeable that the peak concentration of RIF in plasma is normally higher than 5 mg/L [1,7,8]. Therefore, the carry-over should be eliminated to increase the throughput of this analytical method in routine practice.

Contamination and carry-over are common encountered problems with LC–MS/MS analyses [9]. First, the contamination may occur during the sample preparation which is normally related to the extraction procedure. Second, contamination can be generated due to the auto-sampler carry-over. Third, due to secondary interactions in the column a column memory effect may be induced [10]. Dealing with the carry-over requires the combination of systemic and logical investigation [9].

Therefore the aim of the study was to detect and eliminate the carry-over and make the method of analysis more suitable for routine analysis.

2. Methods

2.1. Troubleshooting the carry-over

An effort to detect and eliminate the carry-over from the auto-sampler and column was tried. A more thorough auto-sampler (e.g.

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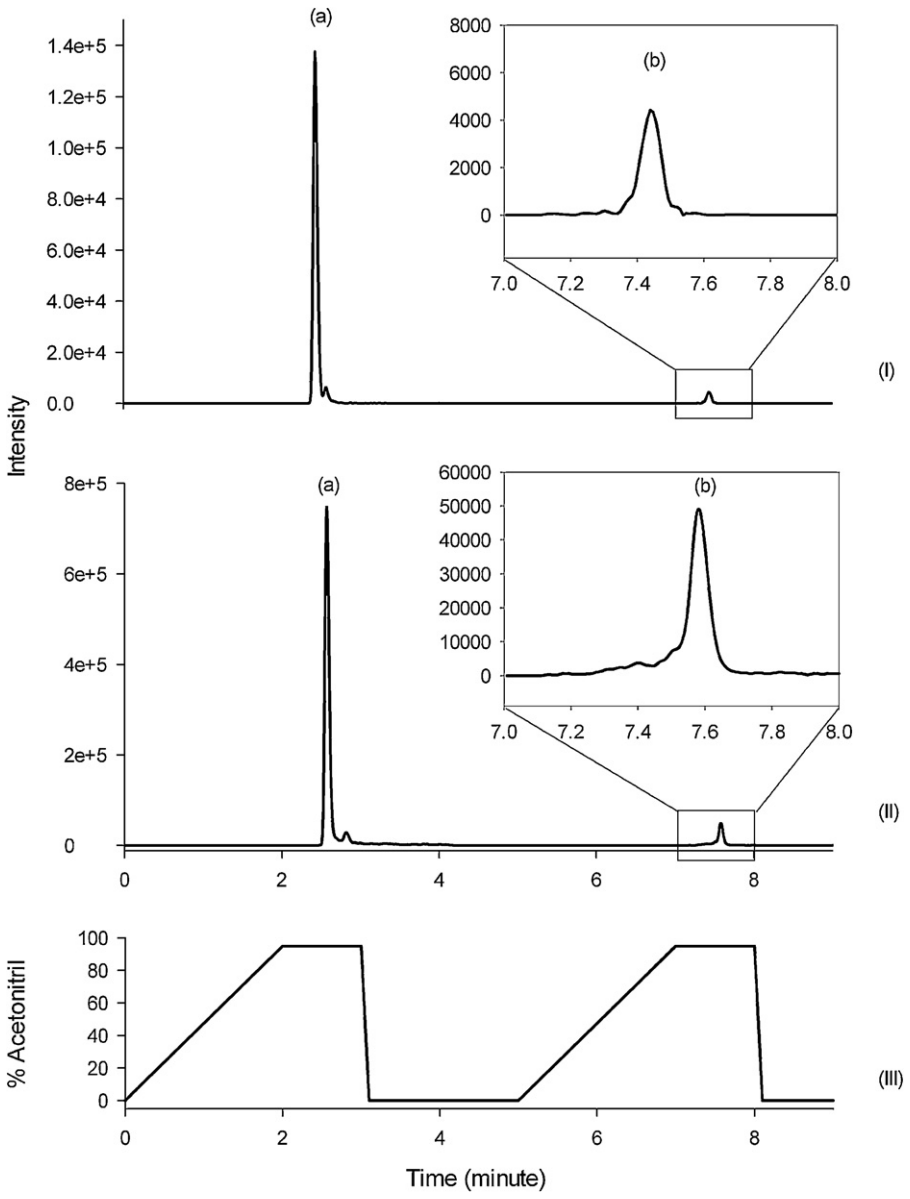

Fig. 1. Chromatogram of the elution of an ULOQ sample using a "duplicated" solvent gradient: (I) 25-desacetylrifampicin; (II) Rifampicin; (III) the "duplicated" gradient; (a) peaks of the first elution period and (b) peaks due to the memory effect.

needle, needle tube and needle seat) flushing and washing procedures using different solvents (acetonitrile, isopropanol and their mixtures) were tested. If the auto-sampler flushing and washing provided no improvement, the carry-over from the column was examined by using a "duplicated" solvent gradient. The previous solvent gradient was performed in 3.5 min as follows: 0–2 min: ACN from 0% to 95%, water from 95% to 0%; 2–3 min: ACN 95% and water 0%; 3–3.1 min: decrease ACN to 0% and keep eluting with water 95% until 3.6 min. The aqueous buffer (ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoroacetic anhydride 2 mg/L, pH 3.5) was kept at 5% during the gradient [5]. The "duplicated" solvent gradient consisted of two gradients, as described before, combined in one analysis. In this way an injection is eluted by the first gradient, while the second gradient is performed without sample injection (Fig. 1). A high concentration sample at ULOQ level (upper limit of quantification; RIF: 30 mg/L; Dac-RIF, CLR and 14OH-CLR: 10 mg/L) was injected and eluted.

To estimate the carry-over effect, 3 samples including a LLOQ (lower limit of quantification RIF; Dac-RIF: 0.15 mg/L; CLR, 14OH-CLR: 0.05 mg/L), a ULOQ and a blank sample were subsequently

Table 1
The concentrations and the inter-assay variation of the calibration curves ($n = 3$).

Component	Calibration concentration (mg/L)	Slope ± SD	Intercept ± SD	Correlation coefficient (R)
CLR	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.421 ± 0.0065	0.0008 ± 0.0064	0.9974
14OH-CLR	0.05, 0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.269 ± 0.0031	−0.0020 ± 0.0029	0.9985
RIF	0.15, 0.45, 1.5, 3.0, 9.0, 15.0, 24.0, 30.0	0.449 ± 0.0081	−0.014 ± 0.0032	0.9971
Dac-RIF	0.15, 0.5, 1.0, 3.0, 5.0, 8.0, 10.0	0.058 ± 0.0012	−0.0026 ± 0.0005	0.9957

injected into the LC–MS/MS system. The carry-over was defined as the percentage of responses from the blank sample to the respective LLOQ sample.

2.2. Method validation

Two different stock solutions for the calibration and quality control samples were prepared in methanol:water (1:1, v/v) containing the following concentrations: RIF: 600 mg/L; Dac-RIF, CLR, and 14OH-CLR: 200 mg/L. Subsequently, the stock solutions were diluted ten times to produce working stock solutions. All stock solutions were stored at 4 °C. Calibration samples (Table 1) and quality control samples at levels of LLOQ, LOW, MED, HIGH and over the calibration curve (OC) (Table 2) were prepared by mixing appropriate amounts of stock solutions or working stock solutions with blank human plasma (received from the Hematology department of UMCG). The added volume of stock solution was less than 5% of the total sample volume.

A plasma volume of 10 μL was transferred into a glass vial with 750 μL of protein precipitation solution, which consists of cyanoimipramine as internal standard in ACN:MeOH (21:4, v/v), The sample was vortexed for 1 min and then stored for 30 min at −20 °C to accelerate the protein precipitation. After 1 min of vortexing and 5 min of centrifuging at 11,000 rpm, 5 μL of supernatant was injected onto the Hypurity C18 column (50 mm × 2.1 mm × 5 μm). For the detection of the analytes a Thermo Fisher triple Quadrupole detector was used. The MS/MS conditions were defined in the previously published method of de Velde et al. [5].

Each day of a 3-day validation, a calibration curve and a set of quality control samples were analyzed. Linear regression weighted by $1/X^2$ was used to construct the calibration curve. For determination of accuracy, precision, and dilution integrity, quality control samples were prepared and measured in 5-fold. Within-run, between-run, and overall bias and coefficient of variation (CV) were calculated using a 1-way ANOVA. Maximum tolerated bias and CV was 20% for the LLOQ and 15% for the other validation concentrations [11].

3. Results and discussion

3.1. Trouble shooting the carry-over of the analysis method for RIF and Dac-RIF

The carry-over may come from the auto-sampler, the switching system or the LC column [10]. No improvement in terms of carry-over was attained by using different kinds of flushing and washing programs and solvents to clean the auto-sampler system. Interestingly, the chromatography of "duplicated" solvent gradient presented a significant column memory effect of RIF and Dac-RIF (Fig. 1). Because the "duplicated" solvent gradient bypasses the auto-sampler and the switching system during the elution period, it is suggested that the persisting "carry-over" in the previously published method resulted from column memory effect.

To eliminate the column memory effect, several gradients were tested using longer eluting periods. Despite of these efforts the memory effect was persistent and the carry-over peaks maintained at about 2% and 4% of the main peaks for Dac-RIF and RIF, respectively. With such high carry-over, the analytical bias of low concentration sample could be dramatically influenced if the previous sample is at high concentration. Injecting several blank samples gradually reduced the carry-over peak yet increased the time of analysis. Extending the elution up to 6 min and increasing the acetonitrile elution phase reduced the carry-over up to 0.7% and 0.2% for RIF and Dac-RIF, respectively. However, short runtimes were

Table 2
The accuracy, precision and the dilution integrity ($n = 5$).

	CLR					14OH-CLR					RIF					Dac-RIF				
	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC	LLOQ	LOW	MED	HIGH	OC
Nominal concentration (mg/L)	0.05	0.15	5.0	8.0	20.0	0.05	0.15	5.0	8.0	20.0	0.15	0.45	15.0	24.0	60.0	0.15	0.5	5.0	8.0	20.0
Accuracy (% bias)	−2.0	1.2	−2.0	1.6	−2.0	11.0	5.1	−0.4	6.0	−2.0	0.3	−9.6	−0.9	1.0	3.0	6.5	−9.1	2.5	10.6	4.0
Within-run precision (% CV)	7.7	3.9	2.1	4.8	5.7	7.6	6.5	2.8	2.7	3.7	5.5	4.9	3.1	3.4	5.3	9.9	6.3	1.7	3.1	4.3
Between run precision (% CV)	8.8	0.0	2.9	4.0	0.0	1.9	2.8	3.6	4.8	0.8	4.7	0.0	0.0	2.8	3.6	0.0	0.0	4.6	4.9	2.6

OC, over the calibration curve.

important to ensure a high throughput of a routine analysis. Moreover, increasing the ULOQ of the original method was preferred for RIF and Dac-RIF to minimize re-analysis of over the curve patient samples. For this purpose, carry-over should be further minimized and therefore another approach should be introduced.

The method published by de Velde et al. used a Hypurity Aquastar C18 column (50 mm × 2.1 mm × 5 μm) for liquid chromatography [5]. The polar end-capped phase added in this column might be the explanation for the observed carry-over. RIF and Dac-Rif may interact with the polar end-capped stationary phase resulting in the column memory effect. For that reason a Hypurity C18 column without polar end-capped phase was tested. Using this column, the carry-over effect observed in an analysis of the first blank sample followed after an ULOQ was dramatically reduced to less than 0.08% for all four substances. With some adjustment of the solvent gradient program, the elution time was shortened to 3 min while the peak shapes remained good and the carry-over was excluded. With this finding, the linear analytical range of all four substances could be extended: RIF: 0.15–30 mg/L; Dac-RIF: 0.15–10 mg/L; CLR and 14OH-CLR: 0.05–10 mg/L.

3.2. Method validation

The method showed good linearity for all four analyzed substances. The equation of the calibration curves and the correlation coefficients are presented in Table 1. In each assay, the deviations of the calibration samples to the linear calibration curves were less than 20% for the lowest concentration and 15% for the other concentrations. It is noticeable that the method was validated with larger analytical ranges than the method published by de Velde et al. On the one hand, no blank injection was needed to exclude the carry-over. On the other hand, the higher ULOQ levels for RIF and Dac-RIF assure the analysis of higher concentrations without the need of diluting and re-analysis of the samples. As a consequence, the new method is more practical for the analysis of real patient plasma samples.

The reproducibility presented as bias and CV were according to the FDA guidelines [11]. All the bias and CV values were less than 20% at LLOQ level and less than 15% at the other QC levels. Diluting the over curve concentration sample influenced neither the accuracy nor the precision of the validated method (Table 2).

4. Conclusions

With the adapted method the carry-over is eliminated and blank sample injections to reduce carry-over have become redundant. The method was re-revalidated and showed to be more practical in routine analysis.

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